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Seasonality in Lipid Content of the Demosponges *Halichondria panicea* and *H. bowerbanki* at Two Study Sites in Temperate Danish Waters

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This study relates the lipid content of two marine filter-feeding demosponges, *Halichondria panicea* and *H. bowerbanki* to the seasonal availability of their suspended food, mainly free-living bacteria and phytoplankton at two study sites in Danish waters. The aim was to investigate if the lipid content of sponges is linked to food availability and season, and to what extent free-living bacteria are available in starvation periods where the phytoplankton biomass is low. The highest concentrations of bacteria were observed during summer when also chlorophyll *a* concentrations were high, and therefore bacteria and phytoplankton were available in similar ratio at all seasons. Bacterial cell carbon was estimated to contribute 2.9 and 4.6% compared to phytoplankton cell carbon in the food at the two sites, respectively, and free-living bacteria were available only as a minor food source at all seasons. Highest lipid contents (29.5% of sponge dry weight) were seen in *H. panicea* at the site with lowest food availability, while the lipid content of *H. bowerbanki* was 11.5% of dry weight. No seasonal variations in lipid content as fraction of sponge dry weight were observed, and the lipid content was not affected by food availability or starvation. It remains unclear why the lipid-content levels at the two study sites were conspicuously different.

Keywords: free-living bacteria, phytoplankton, fatty acids, filter-feeding, seasonality, lipids, Porifera

INTRODUCTION

Halichondria is a common demosponge in for example coastal Dutch (Hummel et al., 1988), German (Barthel, 1986, 1989), Polish (Chojnacki, 2000), and Danish waters (Riisgård et al., 1993; Olesen and Weeks, 1994). Sponges are sessile filter-feeders, evolutionary adapted to nourish on a dilute suspension of microscopic food particles, mainly phytoplankton and free-living bacteria (Riisgård et al., 2016). Food uptake is low in autumn and winter (Koopmans et al., 2015) when low phytoplankton biomass may result in starvation and temporarily decreasing condition index, i.e., the ratio of organic to inorganic matter (Barthel, 1986; Lüsow et al., 2019). The seasonal role of possible energy-storage compounds in sponges is largely unknown. Analysis of the biomass composition of *H. panicea* from Kiel Bight revealed glycogen and lipid contents of 10–30 mg per g ash-free dry weight and 80–140 mg per g ash-free dry weight, respectively (Barthel, 1986).

Neither the lipid, nor the glycogen content seemed, however, to be affected by season (Barthel, 1986). Glycogen is present only in low quantities (<1–3.7% of the dry weight), while lipids may constitute up to 17.3% of the dry weight in demosponges (Stone, 1970; Elvin, 1979). Potentially, lipids may therefore pose the largest energy-storage potential in demosponges, but this has hardly been studied since sponges have no organs or real tissue that may store fat (Rod'kina, 2005).

Sponges contain the greatest diversity of fatty acids among aquatic animals (Rod'kina, 2005), including a variety of long chain (>C22) fatty acids, which are synthesized by elongation of fatty acids from the diet (Carballeira et al., 1986; Rezanka and Sigler, 2009) and used as structural components in cellular membranes (Rod'kina, 2005). Short chained C16 and C18 fatty acids may also be abundant in *H. panicea* and other sponges (Lawson et al., 1984; Rod'kina et al., 2003, Rod'kina, 2005). Palmitic acid (C16:0) and stearic acid (C18:0) account for 1.5–33.0% of the fatty acids in most sponges (Rod'kina, 2005). These fatty acids can be acquired directly from the diet, but their metabolic roles in sponges have not attracted much attention. Sponges also contain filter-feeding trapped phytoplankton and free-living bacteria (Thomassen and Riisgård, 1995) as well as various symbiotic microorganisms (Lee et al., 2001; Hentschel et al., 2006), which also contribute to the lipids that can be extracted from sponges (Rod'kina, 2005).

Sponges efficiently retain free-living bacteria and other particles down to about 0.1 µm in diameter (Fjerdingsstad, 1961; Reiswig, 1971, 1975; Langenbruch, 1983; Leys et al., 2011) and are thus more efficient in retaining such small particles than other co-occurring marine filter-feeding animals, such as, e.g., blue mussels (Lüskow and Riisgård, 2018). The importance of bacteria versus phytoplankton has therefore been debated (see, e.g., Koopmans et al., 2015; Riisgård et al., 2016) and bacteria contribute approximately 20% of the diet of sponges in Danish waters (Lüskow et al., 2019). Since odd numbered long chain fatty acids, stemming from elongation of shorter chain odd numbered fatty acids of bacterial origin can be found in sponges, including *H. panicea* from coastal Dutch waters, bacteria certainly play a nutritional role (Carballeira et al., 1986; Koopmans et al., 2015).

Coastal seawater contains 10^6 to 5×10^6 free-living bacteria ml⁻¹ (Azam et al., 1983; Fenchel, 2008). Higher concentrations may also occur (Andersen and Sørensen, 1986) and vary with season (Bernbom et al., 2011; Lüskow et al., 2019; Richert et al., 2019). It is generally observed that the concentration of free-living heterotrophic bacteria living on dissolved organic matter lost from the phytoplankton is positively correlated with the concentration of chlorophyll *a* in marine environments (Azam et al., 1983; Bird and Kalff, 1984; Guererro et al., 1996; Lüskow et al., 2019; Richert et al., 2019), apparently with the tendency that the bacterial biomass make up a larger proportion of the total suspended biomass when phytoplankton biomass is low (Bird and Kalff, 1984; Pedrós-Alió et al., 1999).

This study relates the lipid content of sponges to the seasonal availability of free-living bacteria and phytoplankton at two study sites in temperate Danish waters; of which one is characterized by relatively low phytoplankton biomass and the presence of the common breadcrumb sponge *H. panicea*, and the second

by relatively high phytoplankton biomass and the presence of a closely related species, *H. bowerbanki*. The aim was to link the lipid content of sponges to seasonal food availability with particular emphasis on the importance of free-living bacteria in order to achieve a better understanding of their nutritional role for sponges.

MATERIALS AND METHODS

Collection and Storage of Sponges

Halichondria panicea (Fleming, 1828) was regularly collected in the inlet to Kerteminde Fjord, Denmark (55°26'59.1"N 10°39'41.0"E), while *H. bowerbanki* (Burton, 1930) was collected in Frederik den VII's Kanal, an excavated harbor channel connected to Limfjorden, Denmark (56°57'51.1"N 9°14'27.0"E) between October 2016 and September 2017. Five specimens were cleaned of epifauna, weighed and stored separately in plastic bags at -20°C until analysis. Specimens were identified based on spicule morphology as *H. panicea* in Kerteminde Fjord and as *H. bowerbanki* in Frederik den VII's Kanal.

Dry Weight and Lipid Content

The wet weight of sponge specimens was measured after water at their surface had been removed by a napkin. Dry weight was measured after drying for 24 h at 100°C. Ash free dry weight was measured after incubation for 6 h at 500°C. Concentrations of carbon present in sponges were estimated using a conversion factor of 0.142 mg C per µg sponge dry weight (Thomassen and Riisgård, 1995). Lipid contents were quantified in lyophilized subsamples of sponge tissue (approximately 1 g) after 24 h of extraction in chloroform/methanol and subsequent evaporation of the solvent as described by Pleissner and Eriksen (2012). Fatty acids were subsequently transmethylylated in 3 ml CH₃OH:HCl:CHCl₃ (10:1:1 v/v/v) at 90°C for 1 h, and the fatty acid methyl esters were extracted in hexane and analyzed using GC-FID, as also described by Pleissner and Eriksen (2012).

Chlorophyll *a* and Bacterial Concentrations

In the inlet to Kerteminde Fjord chlorophyll *a* concentrations were measured using an YSI 650 hand held fluorimeter (Yellowstone Scientific Instruments) at 1 m depth and 1 m away from the harbor wall. In Frederik den VII's Kanal, phytoplankton was trapped on GF/F glass microfiber filters (Whatman) by vacuum filtration of 1 l of water sampled 20 cm below the surface at calm weather conditions. Chlorophyll *a* was extracted from the filters for 24 h at 4°C in 80% ethanol and its concentration was measured spectrophotometrically as described by Arvola (1981). Concentrations of carbon present in the phytoplankton were estimated using a conversion factor of 0.04 mg C per µg of chlorophyll *a* (Li et al., 2010).

The concentration of free-living bacteria was quantified in water sampled at the same time and position as chlorophyll *a* was measured. Bacterial cells were stained with either DAPI or acridine orange and counted under the microscope using

either UV or blue light for excitation. Concentrations of carbon present in the suspended bacterial cells were estimated using a conversion factor of 7.8×10^{-15} g C per bacterial cell (Ferguson and Rublee, 1976).

Weight-Specific Chlorophyll *a* Content in Sponge Tissue

Sponges sampled at the inlet to Kerteminde Fjord were separated into two groups. One group of sponges was used for immediate quantification of chlorophyll *a* in the freshly collected sponge tissue. The second group was placed in bio-filtered (by blue mussels) and aerated seawater (the water was changed every 24 h, ambient salinity) and starved for up to 77 h before chlorophyll *a* was extracted and quantified. The sponges were cut into pieces of 0.33–1.80 g ($n = 10$) and placed in 8 ml 96% ethanol at 4°C. After 24 h the samples were whirl-mixed for 1 min and the sponge remains withdrawn. The extract was centrifuged for 10 min at 3,000 rpm. Chlorophyll *a* was calculated as the difference between

absorbance measurements at 665 and 750 nm. The wet weight-specific chlorophyll *a* content in the sponge tissue was calculated by relating the chlorophyll *a* contents in the extracts to the sponge wet weights.

RESULTS

Seasonality of Food Availability

Concentrations of chlorophyll *a* were generally lower in the inlet to Kerteminde Fjord ($1\text{--}6 \mu\text{g l}^{-1}$) than in Frederik den VII's Kanal ($2\text{ to }15 \mu\text{g l}^{-1}$). Bacterial concentrations were comparable, ranging from a minimum of 2×10^5 cells ml^{-1} at both sites to 1.3×10^6 cells ml^{-1} in the inlet to Kerteminde Fjord and 1.5×10^6 cells ml^{-1} in Frederik den VII's Kanal (Figures 1A,C). Bacterial concentrations were generally highest when also the chlorophyll *a* concentration was high (Figure 1B), and bacteria and phytoplankton were primarily available from

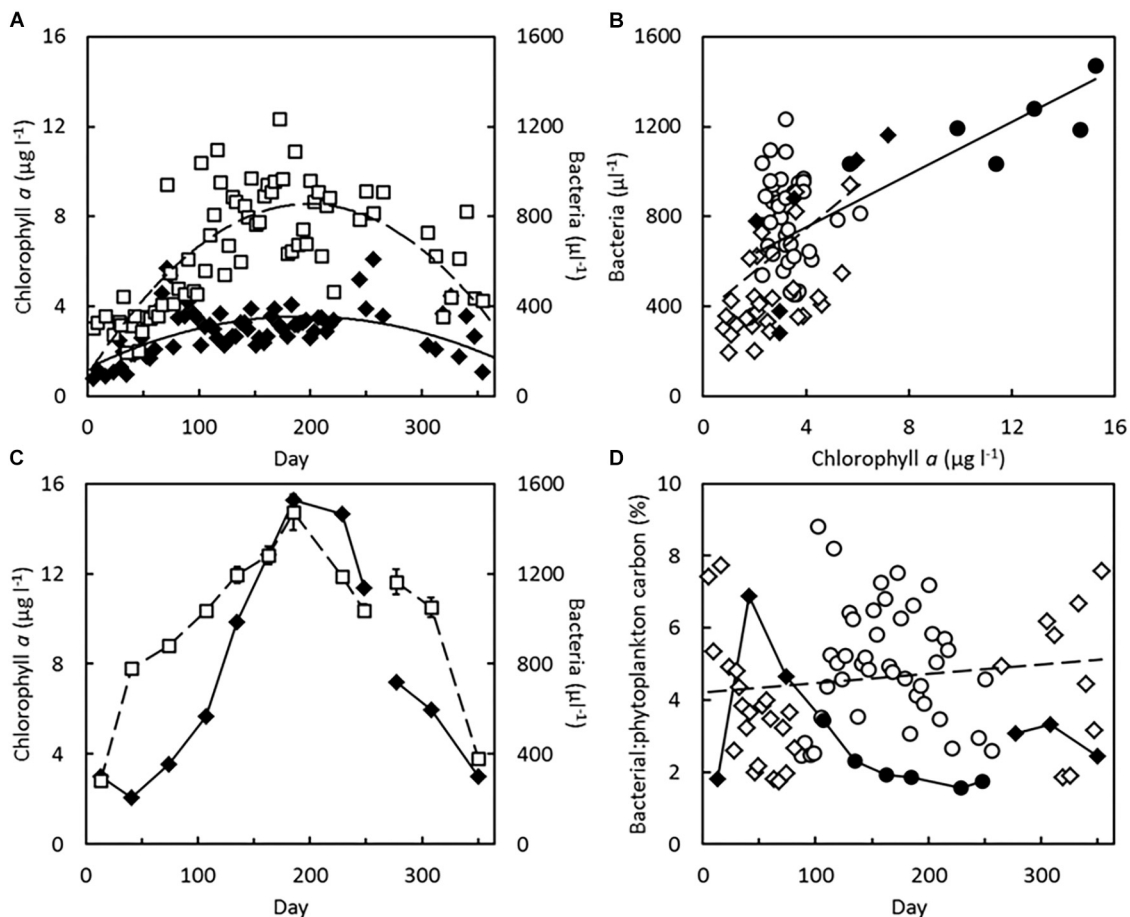


FIGURE 1 | (A,C) Chlorophyll *a* (◆, —) and bacterial (□, —) concentrations in the water column measured over 1-year periods. **(A)** Inlet to Kerteminde Fjord, January 28, 2016 to January 30, 2017. Data redrawn from Lüskow et al. (2019), curves drawn by eye. **(C)** Frederik den VII's Kanal, October 4, 2016 to September 5, 2017. Error bars indicate SEM of three samples. **(B)** Relationship between concentrations of bacteria and chlorophyll *a* in the inlet to Kerteminde Fjord [○ (summer), ◇ (winter), —], $r^2 = 0.16$ and in Frederik den VII's Kanal [● (summer) ◆ (winter), —], $r^2 = 0.63$. **(D)** Ratio of concentrations of cellular carbon in phytoplankton and bacteria for 1-year periods in the inlet to Kerteminde Fjord [○ (summer), ◇ (winter), —], $r^2 = 0.02$ and in Frederik den VII's Kanal [● (summer), ◆ (winter), —]. Summer period from spring equinox (Day 80) until autumn equinox (Day 264), winter period the remaining part of the year.

spring to autumn. On average, the estimated concentrations of cell carbon in phytoplankton and free-living bacteria were $123 \pm 44 \mu\text{g C l}^{-1}$ in the inlet to Kerteminde Fjord and $322 \pm 193 \mu\text{g C l}^{-1}$ in Frederik den VII's Kanal, with average contributions from bacteria of 4.6 ± 1.8 and $7.7 \pm 2.8 \mu\text{g C l}^{-1}$, respectively, at the two sites. The ratio between estimated concentrations of carbon present in bacteria and phytoplankton is shown in **Figure 1D**. The phytoplankton always contributed the majority of the suspended cell carbon. In the inlet to Kerteminde Fjord bacteria contributed on average 4.6% of the cell carbon of phytoplankton. The relationship between bacteria and chlorophyll *a* concentration was generally higher during the summer period, from spring equinox (Day 80) until autumn equinox (Day 264) than during the winter period (**Figure 1D**). Bacteria in Frederik den VII's Kanal accounted for 2.9% of the cell carbon, with the highest relative contribution in February and March (5–7%), when phytoplankton biomass was at a minimum.

Lipid Content in Sponges

Sponges in the inlet to Kerteminde Fjord had an average dry weight content of 8.7% of the wet weight, unaffected by season while sponges in Frederik den VII's Kanal had dry weight content of approximately 10% of the wet weight in October to February. From March to September, the average dry weight content of these sponges increased and peaked at around 20% of the wet weight. In October to November 2016, sponges in Frederik den VII's Kanal had a condition index of 1.16 ± 0.17 ($n = 6$), which in January to February 2017 decreased to 0.53 ± 0.07 ($n = 6$). In April to May 2017, the condition index was 0.58 ± 0.09 ($n = 6$). The dry-weight specific lipid content in sponges from both sampling sites remained constant over the year and was considerably higher in sponges in the inlet to Kerteminde Fjord than in sponges in Frederik den VII's Kanal (**Figure 2**).

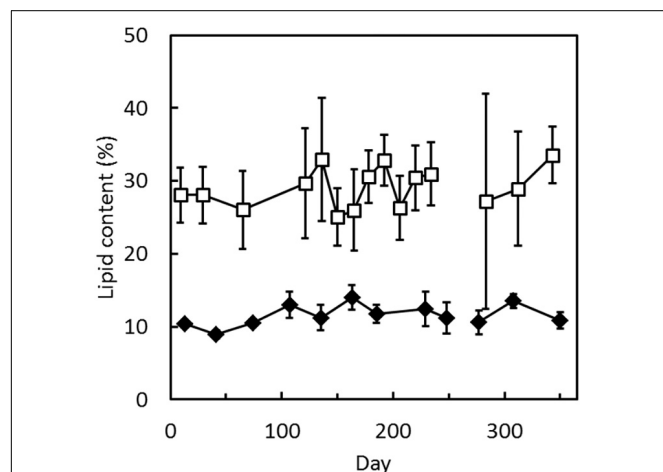


FIGURE 2 | *Halichondria panicea* and *H. bowerbanki*. Lipid content as fraction of dry weight (□) in *H. panicea* from the inlet to Kerteminde Fjord, October 19, 2016 to September 9, 2017 and in *H. bowerbanki* from Frederik den VII's Kanal, October 4, 2016 to September 5, 2017 (◆). Error bars indicate SEM from 3 to 5 specimens.

Composition of Short-Chain Fatty Acids in Sponge Lipids

Palmitic and stearic acid were the most abundant short-chain fatty acids in sponges from both sampling sites (**Figure 3**), as judged from gas chromatograms. The contents of these two fatty acids were variable, but in most specimens, each fatty acid made up less than 10% of the total lipid content in the sponges. They did, however, always co-occur in a C16:C18 ratio of 1.2–1.3 (**Figure 3A**). Other fatty acids were also present in the sponges along with a number of unidentified lipophilic compounds, each contributing less than 1% of the total lipid content.

Specific Chlorophyll *a* Content in Sponges

The decrease of chlorophyll *a* in sponges sampled in the inlet to Kerteminde Fjord was followed after they were transferred to seawater cleared from phytoplankton (**Figure 4**). Chlorophyll *a* was cleared from the sponge tissue at a specific rate of 0.017 h^{-1} , corresponding to a half-life of approximately 40 h. Initially, the sponges contained $17 \mu\text{g}$ chlorophyll *a* per g wet weight, corresponding to a phytoplankton carbon contribution of approximately 0.5% of the organic carbon.

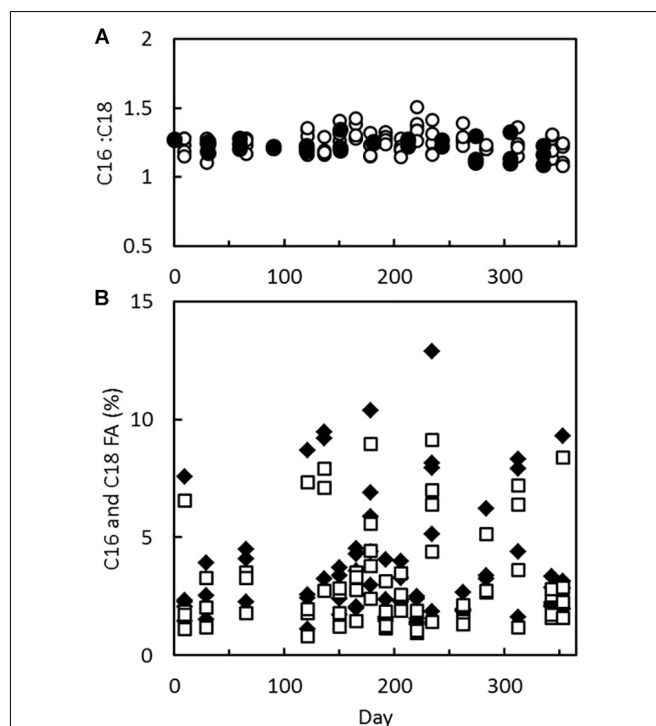


FIGURE 3 | *Halichondria panicea* and *H. bowerbanki*. **(A)** Ratio of palmitic acid and stearic acid, C16:C18 in *H. panicea* from the inlet to Kerteminde Fjord, October 10, 2016 to September 9, 2017 (●) and in *H. bowerbanki* from Frederik den VII's Kanal, October 4, 2016 to September 5, 2017 (○). **(B)** Fraction of palmitic acid, C16:0 (◆) and stearic acid, C18 (□) in lipids of *H. panicea* from the inlet to Kerteminde Fjord, October 4, 2016 to September 9, 2017.

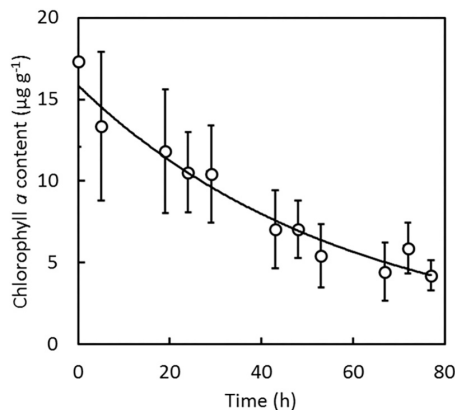


FIGURE 4 | *Halichondria panicea*. Wet-weight specific chlorophyll *a* content in sponge tissue after transfer from the field to bio-filtered seawater without phytoplankton. Data points show average values \pm SD of 10 individual specimens. Regression curve is a first order decay with a rate constant of 0.017 h^{-1} , $r^2 = 0.94$.

DISCUSSION

The concentrations of chlorophyll *a* and free-living bacteria varied over the season at both study sites. Generally, chlorophyll *a* concentrations were lower in the inlet to Kerteminde Fjord ($3 \mu\text{g l}^{-1}$ on average) than in Frederik den VII's Kanal ($8 \mu\text{g l}^{-1}$), while concentrations of bacteria stayed within similar concentration ranges at both sites (Figures 1A,C). Although food availability was highest in Frederik den VII's Kanal, the specific lipid content was highest in *Halichondria panicea* from the inlet to Kerteminde Fjord. The specific lipid content did not show signs of seasonal variation. Bacteria made up a minor fraction of the available biomass all year round and have thus played a minor role as food for the sponges, in agreement with Lüskow et al. (2019).

In Frederik den VII's Kanal, there was a correlation between concentration of bacteria and biomass of phytoplankton (measured as concentration of chlorophyll *a*) over the 1-year period ($r^2 = 0.63$, Figure 1B). In February and March (Days 41 and 74), bacteria contributed more to the suspended biomass than in the rest of the year. In the inlet to Kerteminde Fjord, chlorophyll *a* concentrations remained within a narrower concentration range and the correlation between concentrations of bacteria and chlorophyll *a* was weak ($r^2 = 0.16$, Figure 1B). At this site, the ratio between bacteria and chlorophyll *a* was generally higher in summer, when also the primary production and the loss of dissolved organic matter are high (Azam et al., 1983), than in winter (Figure 1D). In 1983, bacterial concentrations were followed from March to November at an open-coast location in the eutrophic Limfjorden (Andersen and Sørensen, 1986). In early spring, bacterial concentrations were below $10^6 \text{ cells ml}^{-1}$, while maximal summer concentrations were up to $15 \times 10^6 \text{ cells ml}^{-1}$. In comparison, the availability of free-living bacteria was therefore relatively low at both present study sites. The estimated bacterial carbon contributions (Figure 1D) were based on a conversion factor of $7.8 \times 10^{-15} \text{ g C per bacterial cell}$ (Ferguson and

Rublee, 1976). Other studies have indicated cell-specific carbon contents of marine bacteria in Danish waters between 7 and $43 \times 10^{-15} \text{ g C cell}^{-1}$ (Fukuda et al., 1998). Thus, bacteria may have contributed between 4.1 and 25.2% of the carbon present in phytoplankton in the inlet to Kerteminde Fjord and 2.6 to 13.8% in Frederik den VII's Kanal.

The lipid content was high in *H. panicea* from the inlet to Kerteminde Fjord (29.5%) compared to earlier reports, while the lipid content in *H. bowerbanki* from Frederik den VII's Kanal (11.5%) was comparable to previously published values (Stone, 1970; Elvin, 1979; Barthel, 1986). No seasonality was observed in the lipid content (Figure 2). It seems to be a general trend that the lipid content of *Halichondria* is independent of season (Barthel, 1986), although negative growth and decreasing condition index indicate that the sponges are starving during autumn and winter (Barthel, 1986; Lüskow et al., 2019). Seasonality was also not observed in the contents of palmitic acid or stearic acid (Figure 3), indicating that the role of these fatty acids is not directly associated with energy metabolism. The high condition index of *H. bowerbanki* from Frederik den VII's Kanal measured in October to November 2016 (1.16 ± 0.17) and the low condition index measured in January to February 2017 (0.53 ± 0.07) were similar to measurements of condition indices of *H. panicea* in the inlet to Kerteminde Fjord (Lüskow et al., 2019) and lipids have therefore contributed more of the organic fraction of the sponges in winter when the condition index was low. It is not clear why changes in condition index were not reflected in the lipid content as fraction of dry weight. Also in the blue mussel *Mytilus edulis*, which is common in the same habitats, there seems to be no clear relationship between lipid content and nutritional status, but in contrast to sponges, blue mussels contain a large pool of glycogen that can be mobilized during starvation periods (Pleissner et al., 2012).

Lipids from bacteria may to a minor extent have contributed to the lipids extracted from the sponges. *Halichondria* belongs to the so-called low microbial abundance (LMA) sponges (Knobloch et al., 2019), which according to Hentschel et al. (2006) contain between 10^5 and 10^6 bacterial cells per g wet weight. With an average dry weight of marine bacteria of $2.3 \times 10^{-14} \text{ g cell}^{-1}$ (Ferguson and Rublee, 1976) and average dry weight contents of 8.7–13.5% in sponges from the inlet to Kerteminde Fjord and Frederik den VII's Kanal, respectively, the bacterial biomass made up a maximum of only 3×10^{-5} to $4 \times 10^{-5}\%$ of the sponge dry weight. Furthermore, Gillan et al. (1988) found that fatty acids from bacteria made up only 56–437 $\mu\text{g g}^{-1}$ (0.005–0.0437%) of the total fatty acids in five tropical sponge species. The contribution of lipids from trapped phytoplankton was also modest, although the sponges did contain measurable quantities of chlorophyll *a* (Figure 4). Chlorophyll *a* decayed in the sponges at a rate of 0.017 h^{-1} after transfer to phytoplankton-free seawater. This has also been observed in *H. panicea* by Thomassen and Riisgård (1995), from whose data it can be estimated that the rate of decay was 0.035 h^{-1} for sponges transferred directly from the field, and 0.031 h^{-1} for sponges fed algal cells in the laboratory prior to transfer to clean seawater. These observations indicate that chlorophyll *a* in *Halichondria* primarily stem from captured, but not yet digested

phytoplankton. Using a conversion factor of 0.04 mg C per μg of chlorophyll *a* (Li et al., 2010), it can be estimated that phytoplankton contributed less than 0.5% to the organic biomass of *H. panicea*. It is also noticeably that palmitic acid and stearic acid were always present in a constant ratio of 1.26 ± 0.08 in *H. panicea* from the inlet to Kerteminde Fjord and 1.22 ± 0.06 in *H. bowerbanki* from Frederik den VII's Kanal (Figure 3). This indicates that the composition of these lipids is under stricter control than would be the case if lipids were derived from a variety of trapped or symbiotic microorganisms.

CONCLUSION

Halichondria panicea and *H. bowerbanki* efficiently retain phytoplankton and bacteria, and since the abundance of bacteria follows the phytoplankton biomass and always make up the smallest fraction of the two food sources, consumption of free-living bacteria cannot explain why the lipid content of sponges remains stable, even when decreasing condition index and low chlorophyll *a* concentrations indicate starvation. *Halichondria* does not rely on accumulated energy reserves during periods of starvation but recycle their own cells during winter, apparently leaving the specific lipid content as a fraction of dry weight constant at all seasons. It remains unclear why the pronounced variation in condition index was not reflected in the lipid content, and why the lipid-content levels at the two study sites were conspicuously different.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

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ETHICS STATEMENT

The study was conducted on marine sponges (invertebrates) and therefore exempted from this requirement.

AUTHOR CONTRIBUTIONS

FL carried out sampling and experimental work in the inlet to Kerteminde Fjord and wrote the first draft of the manuscript in collaboration with NE. KK-M quantified the lipids and carried out fatty acid analysis in collaboration with LP. JT carried out sampling and experimental work in Frederik den VII's Kanal. LP carried out fatty acid analysis in collaboration with KK-M. HR conceived and designed the experiments in collaboration with FL and NE. NE conceived and designed the experiments in collaboration with FL and HR and wrote the first draft of the manuscript in collaboration with FL.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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